expression of dominant-negative ActRIB could lead to inhibited degradation and extension of the activation period, thereby inducing an ACM phenocopy.

Previous studies have suggested correlations between anxiety and depression. Many popularly prescribed antidepressant drugs modulate monoamine neurotransmission, which could take 6–8 weeks to exert effects, and individual drugs are efficacious in only 60–70% of patients. A novel antidepressant, which acts rapidly and safely in a high proportion of patients, would be highly advantageous (Wong and Licinio, 2004). Therefore, activins and the involved signaling pathway could represent novel therapeutic targets for depression, as well as ischemic brain injury. The transgenic mice generated in our laboratory could be useful for screening compounds for newly developed and novel antidepressant drugs (Ageta *et al.*, 2008).

C. Activin is an important factor in adult neurogenesis

Postnatal neurogenesis, which is the production of new neurons in the adult brain, influences a number of physiological roles, including the replacement of damaged neurons, stress responses, and memory formation (Gage, 2002; Kitamura *et al.*, 2009; Kokaia and Lindvall, 2003; Shors *et al.*, 2001; Wang *et al.*, 2004). Several recent reports have demonstrated that neurogenesis is also involved in depression (Malberg *et al.*, 2000; Santarelli *et al.*, 2003).

Bromodeoxyuridine (BrdU), which is a thymidine analog, is incorporated into genomic DNA during S-phase and can be used as a marker of dividing cells. Through double-labeling and immunofluorescent methods utilizing antibodies specific for BrdU and the mature neuronal marker NeuN, newly generated neurons can be easily detected within brain tissue sections (Kuhn *et al.*, 1996).

In 2000, chronic antidepressant treatment was shown to significantly increase the number of BrdU-labeled cells in the hippocampal dentate gyrus and hilus (Malberg *et al.*, 2000). An additional study demonstrated that the disruption of antidepressant-induced neurogenesis actually blocks the behavioral effects of antidepressants (Santarelli *et al.*, 2003). Recently, our laboratory showed that activins are involved in adult neurogenesis (Ageta *et al.*, 2008); FSM and ACM 5-week-old transgenic mice were injected with 75 mg/kg BrdU 3 times per day for 3 days. These mice were sacrificed either 24 h or 4 weeks after the final injection; at 24 h after the final injection, BrdU incorporation was measured in the hippocampal subgranular zone (SGZ), where dividing progenitor cells are located (Gage, 2002). There was no change in the number of BrdU-positive cells in the transgenic mice compared with wild-type mice at 24 h. However, after 4 weeks, the number of BrdU/NeuN-positive cells was significantly decreased in the FSM mice, but there was no neurogenesis effect in the ACM mice.

These results indicated that endogenous activin signals are essential for adult neurogenesis (Ageta *et al.*, 2008). In addition, the level of neurogenesis in FSM/ACM-double transgenic mice was significantly greater than in FSM mice, indicating that reduced neurogenesis in the FSM mice was partially rescued by increased activin expression (Ageta *et al.*, 2008). Similar results have been reported by other groups (Abdipranoto-Cowley *et al.*, 2009). These studies suggested that activin is an important factor for adult neurogenesis.

Follistatin inhibits activins, but it also antagonizes other members of the TGF-β superfamily, namely, GDF11/BMP11 (Gamer *et al.*, 1999) and GDF8/myostatin (Tsuchida, 2004). Because increased neurogenesis was not observed in the activin overexpressing mice (ACM), the possibility of decreased neurogenesis in follistatin overexpressing mice (FSM) due to GDF11 or GDF8 could not be ruled out. However, the likelihood of this is quite low, because of the following: (1) GDF8 is exclusively expressed in skeletal muscle (McPherron *et al.*, 1997; Sharma *et al.*, 1999); (2) GDF11 is mainly expressed in olfactory epithelium (OE) in adult mice; *GDF11* mRNA expression is low in the hippocampus (ALLEN Brain project: http://www.brain-map.org); and (3) GDF11 acts as a negative regulator for neurogenesis in the OE. In fact, GDF11 inhibits production of progenitors and neurons (Wu *et al.*, 2003), which was shown in mice lacking follistatin. In FSM mice, the number of progenitor cells is normal, but survival of newly generated neurons is significantly decreased (Ageta *et al.*, 2008). Therefore, it is likely that activins, not GDF8 and GDF11, regulate adult hippocampal neurogenesis.

Results from our study demonstrated that decreased postnatal neurogenesis, which is due to activin inhibition, results in anxiety-related behavior during adulthood (Fig. 9.8).

Activin treatment in hippocampal cultures suppresses emergence of GAD67(+) GABAergic neurons and increases the percentage of Prox1 (+), dentate granule neurons. In contrast, follistatin treatment increases the percentage of GAD67(+) neurons and decreases the percentage of Prox1(+) neurons (Sekiguchi *et al.*, 2009). These results indicated that activin signaling during postnatal neural development alters neural circuitry composition by regulating the ratio of excitatory to inhibitory neurons. In addition, results have shown that GABAergic neurotransmission is altered in dominant-negative ActRIB transgenic mice (Zheng *et al.*, 2009).

Activins increase synaptic input for each individual spine (Fig. 9.2; Shoji-Kasai *et al.*, 2007), Ca^{2+} influx *via* the NMDA receptor (Kurisaki *et al.*, 2008), ratio of excitatory to inhibitory neurons in newly generated neurons (Sekiguchi *et al.*, 2009), and maintenance of early-phase LTP (E-LTP; see below) (Ageta *et al.*, 2010).

When the summation of excitatory and inhibitory postsynaptic potential from numerous synaptic inputs reaches the triggering threshold, the action

potential propagates through the axon and triggers neurotransmitter release from the presynaptic region. Therefore, these activin-dependent actions positively affect action potential activation; in other words, activins induce activation of the entire neuronal circuit.

Because electroconvulsive therapy (ECT) is used most often to treat severe major depression that does not respond to other treatments (Mukherjee *et al.*, 1994), the resulting activin-dependent changes could positively affect depression. If this were the case, the combination of compounds that enhance activin signals and low-level electroconvulsive stimulation may be suitable for human therapy, because low-level stimulation reduces unpredictable side effects. In addition, the treatment of compounds that enhance activin signals could be utilized to replace ECT.

D. Activin is a key player for maintaining late-phase LTP

LTP, which is thought to underlie learning and memory mechanisms, exhibits two distinct phases, named early-phase LTP (E-LTP) and late-phase LTP (L-LTP). E-LTP persists for several hours and does not depend on protein synthesis, whereas L-LTP persists for weeks and depends on *de novo* RNA transcription and protein synthesis (Abraham *et al.*, 1993; Frey *et al.*, 1988; Nguyen *et al.*, 1994). The formation of LTM also requires *de novo* RNA transcription and protein synthesis (Bourtchuladze *et al.*, 1994; Castellucci *et al.*, 1989; Squire and Barondes, 1973). Therefore, activity-dependent gene expression is expected to play a crucial role in LTM.

To understand the molecular mechanisms of LTM, many studies have isolated a number of neuronal activity-dependent genes, including *inhibin* βA (Andreasson and Worley, 1995; Inokuchi *et al.*, 1996), *vesl-1s/homer-1a* (Brakeman *et al.*, 1997; Kato *et al.*, 1997), *zif268* (Cole *et al.*, 1989), *arc* (Lyford *et al.*, 1995), and *scrapper* (Yao *et al.*, 2007). *Arc* and *zif268*-deficient mice exhibit impaired long-term memory, but not short-term memory (Jones *et al.*, 2001; Plath *et al.*, 2006). Vesl-1S protein is synaptically tagged *via* the ubiquitin-proteasome system (Ageta *et al.*, 2001a,b) and it regulates spinal morphology and synaptic responses (Hennou *et al.*, 2003; Sala *et al.*, 2003). Furthermore, *zif268* and *vesl-1S* knockout mice also exhibit deficient reconsolidation memory processes (see Section IV.E; Bozon *et al.*, 2003; Inoue *et al.*, 2009). These results indicated that neural activity-dependent genes have important roles in the memory process.

Recently, we examined hippocampal dentate gyrus LTP in urethane-anesthetized rats. Results showed that follistatin or antiactivin A antibody inhibits L-LTP formation without affecting E-LTP (Fig. 9.4A). The decay time course is similar to that of animals injected with the protein synthesis inhibitor anisomycin. Activins facilitate E-LTP duration (Fig. 9.4B) (Ageta *et al.*, 2010), and maintenance of CA1 L-LTP, but not E-LTP, in

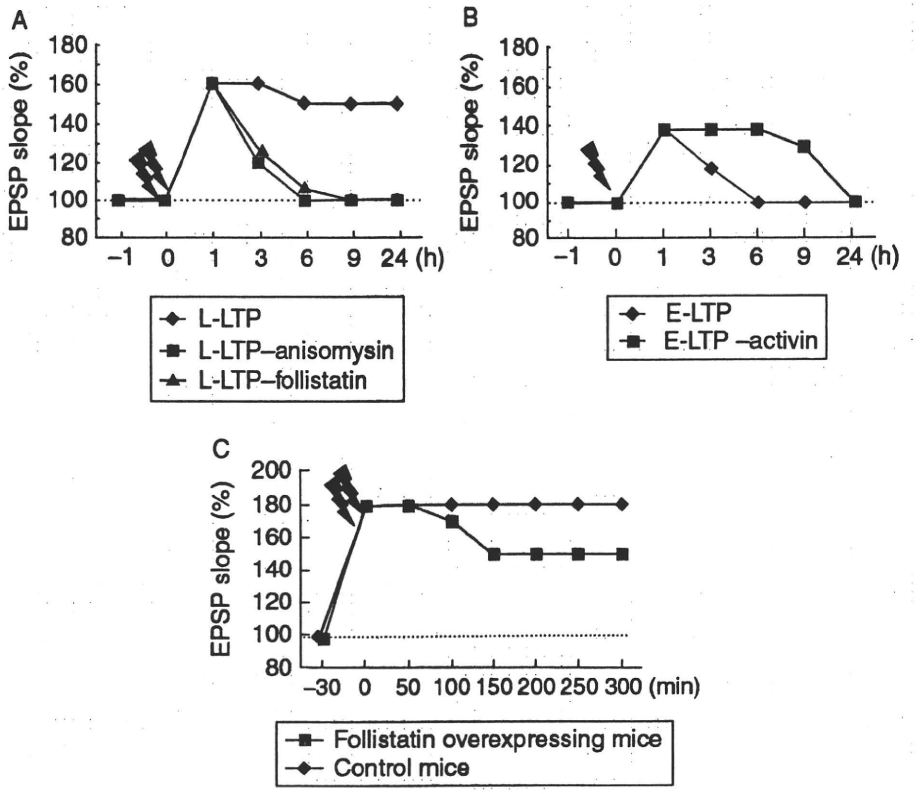

Figure 9.4 Activin is required for L-LTP. (A) Effect of follistatin, antiactivin A, and anisomycin on dentate gyrus LTP persistence in urethane-anesthetized rats. (B) Effect of activin on dentate gyrus LTP persistence in urethane-anesthetized rats. (C) Maintenance of CA1 L-LTP in hippocampal slices from follistatin overexpressing mice and control mice. Two lightning symbols represent strong high-frequency stimulations produced by L-LTP. One lightning symbol represents a weak high-frequency stimulation elicited by E-LTP.

hippocampal slices from follistatin transgenic mice (FBItTA, see below) is significantly reduced compared with control mice (Fig. 9.4C; Ageta *et al.*, 2010).

In the marine snail *Aplysia*, TGF-β induces long-term, but not short-term, facilitation at synapses between sensory and motor neurons (Zhang *et al.*, 1997). Similarly, treatment with TGF-β2, another isoform of TGF-β, affects synaptic strength and induces CREB phosphorylation in rat cultured hippocampal neurons (Fukushima *et al.*, 2007). Therefore, the TGF-β family of proteins, namely, activins and TGF-β1/2, participate not only in development, but also in neuronal plasticity, of the mature CNS.

E. Activin influences reconsolidation and extinction

LTM consists of several distinct processes—acquisition (training), maintenance, and retrieval (recall) phase—through which memory is consolidated. Two recent studies revealed that retrieval of consolidated memory leads to

two opposing processes: one that weakens old memory and another that strengthens it (Eisenberg *et al.*, 2003; Suzuki *et al.*, 2004). The former process is known as "extinction learning." The latter process has recently been referred to as "reconsolidation"; memory could be vulnerable following retrieval, so it is reconsolidated in a protein synthesis-dependent manner (Fig. 9.5; Nader, 2003; Nader *et al.*, 2000; Tronson and Taylor, 2007). The study of reconsolidation has extended to numerous learning tasks, such as fear conditioning test (Nader *et al.*, 2000), Morris water maze (Suzuki *et al.*, 2004), and object recognition test (Kelly *et al.*, 2003) in various species such as crabs (Pedreira *et al.*, 2002), chicks (Anokhin *et al.*, 2002), Medaka fish (Eisenberg et al., 2003), rodents (Nader *et al.*, 2000), and humans (Walker *et al.*, 2003). These results suggest that "reconsolidation" is an evolutionarily conserved memory system. Because there is a possibility that enhanced extinction learning or disrupted reconsolidation could be particularly efficacious when treating strong traumatic memory-related disorders, such as posttraumatic stress disorder (PTSD) or phobias, studies have investigated the molecular mechanisms of these processes. In addition, results from our laboratory recently demonstrated that activins in the brain affect both extinction and reconsolidation (Ageta *et al.*, 2010).

To examine the role that activin plays in fear memory formation, spatiotemporal-regulated activin (ABItTA) and follistatin (FBItTA) transgenic mice were generated (Ageta *et al.*, 2010). When follistatins are continuously expressed in the brain during training, maintenance, and retrieval phases (Fig. 9.6, Exp. A), FBItTA mice are impaired in LTM but not short-term memory (STM). These results were consistent with activin requirements for L-LTP (see above). Furthermore, a 1-week memory test was performed on FBItTA mice to determine how reconsolidation processes are regulated by activin inhibition (Fig. 9.6, Exp. B). When follistatins were expressed in the brain during maintenance and retrieval phases, there was no significant genotype effect on Test 1 between the FBItTA and control mice (Fig. 9.6, Exp. B). In addition, when the same animals were retested for freezing behavior 24 h later (Test 2 in Exp. B), the FBItTA mice exhibited significantly fewer freezing responses compared with the control mice. When follistatin expression was suppressed during all phases in FBItTA mice, significant genotype effects were not observed in either Test 1 or 2. Therefore, inhibition of activin signals during retrieval resulted in suppressed reconsolidation.

Three-week memory testing was also performed on the ABItTA mice, which induces extinction (Fig. 9.7). When activins were upregulated in the brain during maintenance and retrieval phases, there was no significant genotype effect in Test 1 between the ABItTA and control mice (Fig. 9.7, Exp. C). In this experimental paradigm, the freezing level was significantly less in Test 2 compared with Test 1 in control mice. However, there was no significant change between Test 1 and 2 in ABItTA mice.

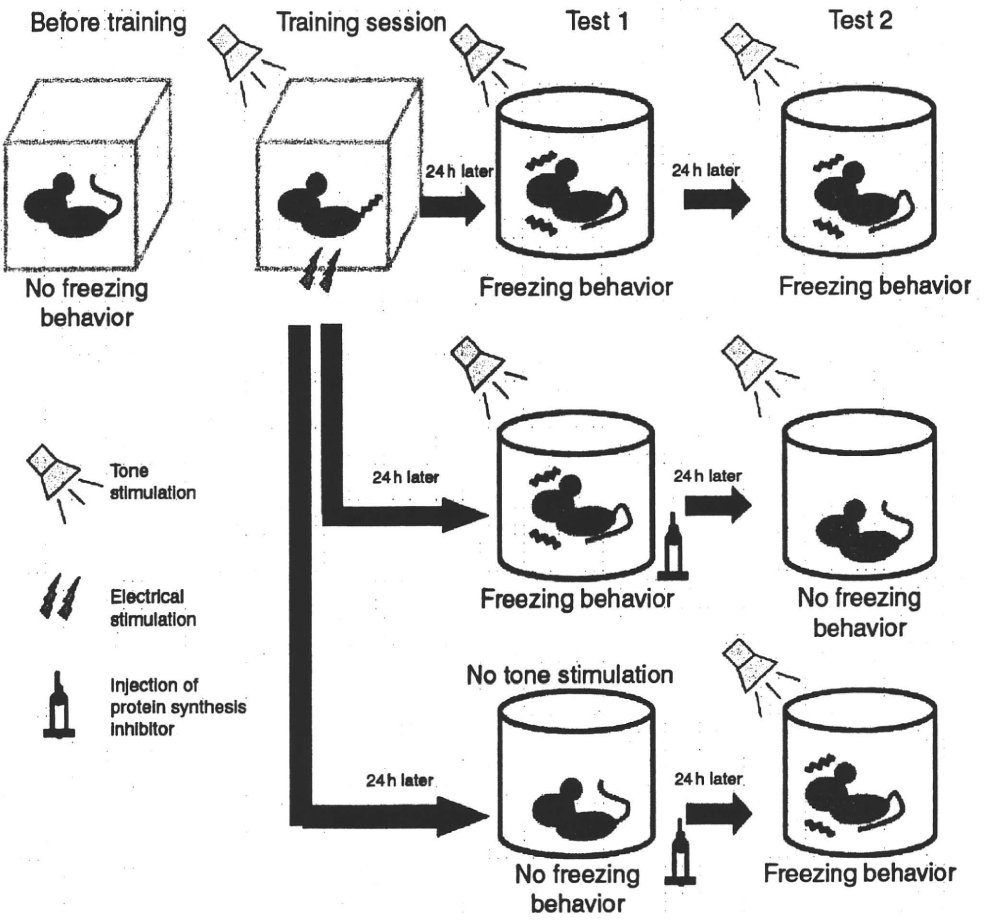

Figure 9.5 Memory reconsolidation in 2000, Nader *et al.* (2000) demonstrated reconsolidation in rats using a cued-fear conditioning test. The illustrations represent the Nader experiment. In brief, rats are placed in chamber A (gray box) and then receive tone and electrical stimulation. After 24 h, rats are placed in chamber B (red columnar box), whose shape is different from that of chamber A, and the rats are exposed to tone (Test 1 in upper row). If the rats associate tone with danger (electrical stimulation), they exhibit freezing behavior. After 24 h, the same rats were retested for freezing behavior (Test 2 in upper line). In the experiment paradigm that triggers reconsolidation process, the rats exhibited freezing behavior again in Test 2 (Test 2 in upper row). In this experimental paradigm, Nader *et al.* demonstrated that intra-amygdala inhibition of protein synthesis, following retrieval of a previously consolidated memory, resulted in amnesia (no freezing behavior) for the retrieved memory (Test 2 in middle row), but not for consolidated memories that were not retrieved (Test 2 in lower row). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)

ABItTA mice also exhibited significantly more freezing than control mice in Test 2. These findings demonstrated that activin upregulation in the brain inhibits the extinction learning.

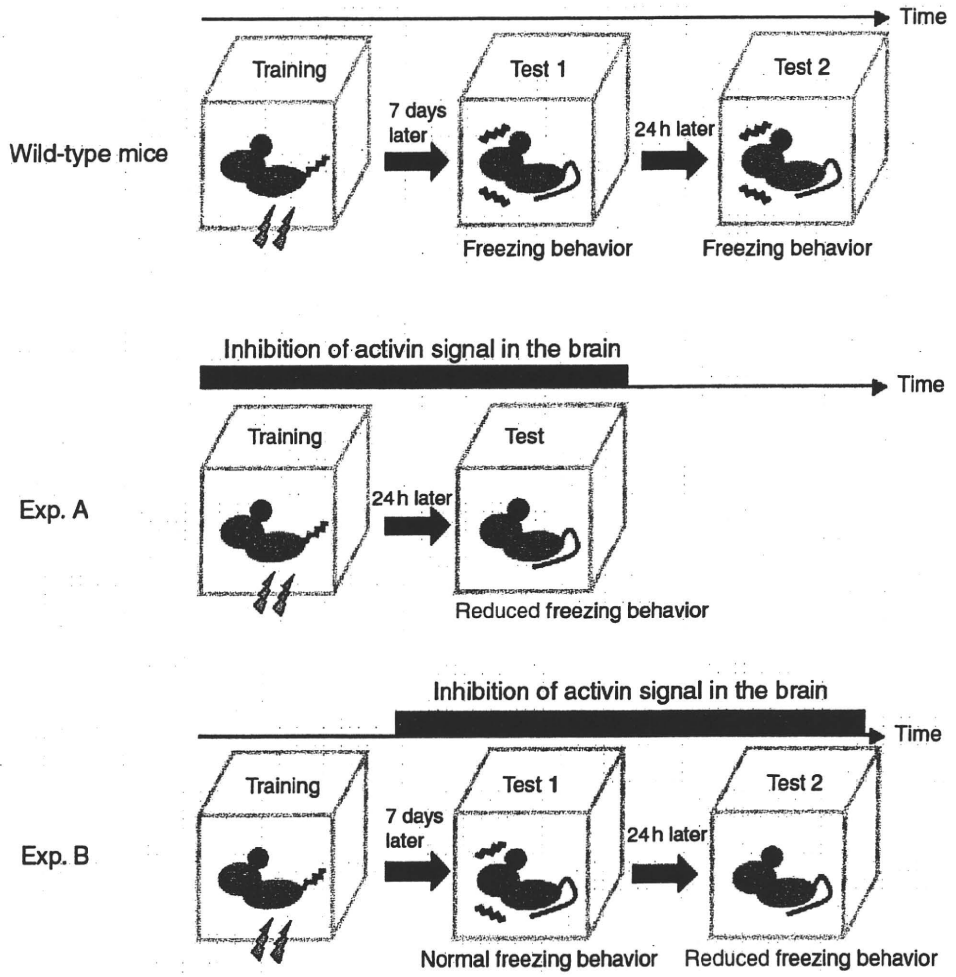

Figure 9.6 Contextual fear-conditioning test triggers reconsolidation in FBItTA mice. Utilizing the contextual fear-conditioning test, mice were placed in chamber A (gray box) and were exposed to electrical stimulation. During the retrieval phase, the mice were placed in the same chamber (Test). If the mice associate the chamber with danger, they will exhibit a freezing behavior during the Test phase. In the experiment paradigm that triggers reconsolidation, wild-type mice exhibited freezing behavior again during Test 2 (Test 2 in upper line). In this experimental paradigm, when mice were injected with protein synthesis inhibitor 30 min after Test 1, the mice exhibited reduced freezing behavior in Test 2. In experiment A (Exp. A, middle row), follistatins were continuously expressed during training, maintenance, and retrieval phases in the brain of FBItTA mice. FBItTA mice exhibited reduced freezing behavior during testing in Exp. A. In experiment B (Exp. B, lower row), follistatins were expressed in the brain during maintenance and retrieval phases in FBItTA mice. FBItTA mice exhibited reduced freezing behavior during Test 2, but not during Test 1, of Exp. B. Two lightning symbols represent strong electrical stimulation.

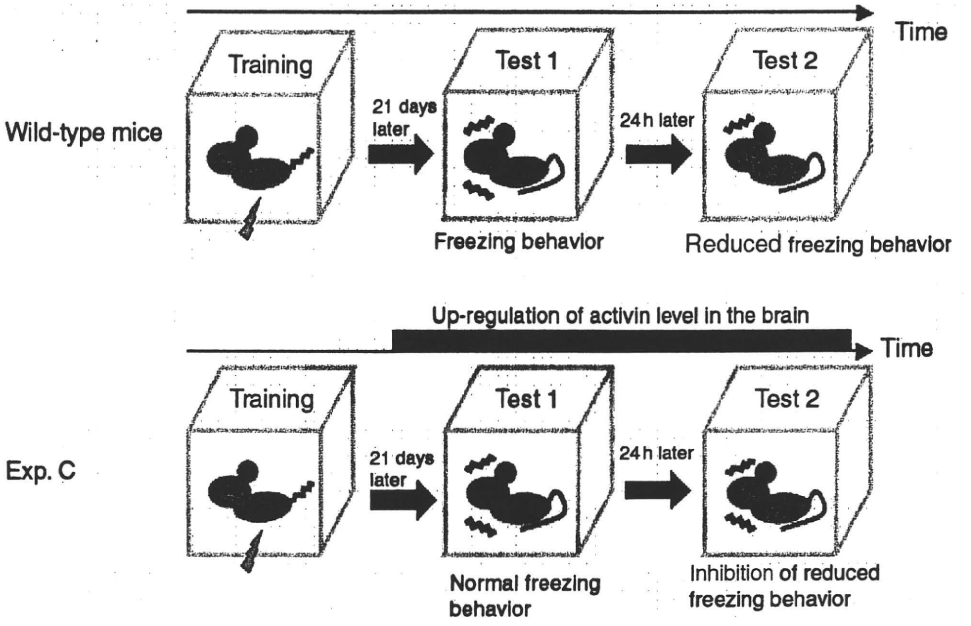

Figure 9.7 **Contextual fear-conditioning test triggers extinction learning in ABItTA mice. In an experiment paradigm that triggers extinction learning, wild-type mice exhibited reduced freezing behavior during Test 2 (Test 2 in upper line). The level of electrical stimulation and length of maintenance phase in Exp. C were different from Exp. B. In experiment C (Exp. C, lower row), activin levels were elevated during maintenance and retrieval phases in the brains of ABItTA mice. ABItTA mice did not exhibit reduced freezing behavior during Test 2 of Exp. C. One lightning symbol represents weak electrical stimulation.**

Results from our studies demonstrated that activin inhibition during memory retrieval suppresses previously consolidated fear memories. Therefore, activin signaling could be a promising target for the treatment of disorders that are based on strong traumatic memories, such as PTSD and phobias.

V. Conclusion and Perspectives

Activins are involved in various brain functions, including spine formation, anxiety, neurogenesis, L-LTP, LTM, extinction, and reconsolidation (Fig. 9.8). Recent studies have also shown that activins exhibit neurotrophic and neuroprotective effects (Hughes *et al.*, 1999; Tretter *et al.*, 2000; Wu *et al.*, 1999). For these reasons, activin-related compounds have therapeutic potential for candidate drugs to treat CNS disorders.

Because antibodies and peptides cannot cross the blood–brain barrier, activins, antiactivin antibodies, and follistatins are not suitable for treating the above-mentioned CNS disorders. Recently, small molecules, such as

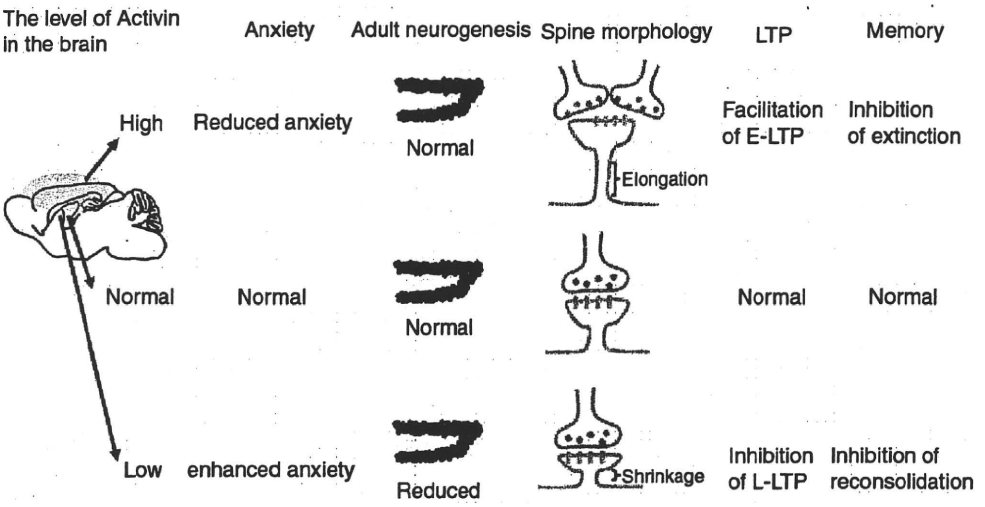

Figure 9.8 **Summary of the role of activin in the brain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this chapter.)**

SB-431542 and GW788388, were identified as specific inhibitors of type I activin receptor-like kinase (ALK) receptors, including ActRIB/ALK4, ALK5, and ALK7 (Inman *et al.*, 2002). In addition to small molecule compounds, orally active ALK5 inhibitors were also developed (Gellibert *et al.*, 2006). If these compounds cross the blood–brain barrier, they may also be useful for treating CNS disorders. Because activin-signaling cascades exist in various peripheral tissues, activin-related compounds could result in unpredictable side effects in various tissues. To minimize side effects, it could be essential to combine the development of novel activin-related compounds with brain-specific drug delivery systems. We believe that the development of activin and follistatin transgenic mice could be useful for these drug developments.

ACKNOWLEDGMENTS

We acknowledge the collaborative studies with Dr. K. Inokuchi at Toyama University/MITILS. This work was partly supported by a research grant (H20-018) on psychiatric and neurological diseases from the Ministry of Health, Labour and Welfare and an intramural research grant (20B-13) for neurological and psychiatric disorders of NCNP.

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Research

Activin plays a key role in the maintenance of long-term memory and late-LTP

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A recent study has revealed that fear memory may be vulnerable following retrieval, and is then reconsolidated in a protein synthesis-dependent manner. However, little is known about the molecular mechanisms of these processes. Activin βA, a member of the TGF-β superfamily, is increased in activated neuronal circuits and regulates dendritic spine morphology. To clarify the role of activin in the synaptic plasticity of the adult brain, we examined the effect of inhibiting or enhancing activin function on hippocampal long-term potentiation (LTP). We found that follistatin, a specific inhibitor of activin, blocked the maintenance of late LTP (L-LTP) in the hippocampus. In contrast, administration of activin facilitated the maintenance of early LTP (E-LTP). We generated forebrain-specific activin- or follistatin-transgenic mice in which transgene expression is under the control of the Tet-OFF system. Maintenance of hippocampal L-LTP was blocked in the follistatin-transgenic mice. In the contextual fear-conditioning test, we found that follistatin blocked the formation of long-term memory (LTM) without affecting short-term memory (STM). Furthermore, consolidated memory was selectively weakened by the expression of follistatin during retrieval, but not during the maintenance phase. On the other hand, the maintenance of memory was also influenced by activin overexpression during the retrieval phase. Thus, the level of activin in the brain during the retrieval phase plays a key role in the maintenance of long-term memory.

[Supplemental material is available online at http://www.learnmem.org.]

Formation of long-term memory (LTM) consists of several distinct processes: acquisition, consolidation, and reconsolidation, through which memory becomes permanent (Nader et al. 2000; Rodrigues et al. 2004; Tronson and Taylor 2007; Kitamura et al. 2009). The prominent feature of LTM is a requirement for RNA and protein synthesis for consolidation and reconsolidation (Squire and Barondes 1973; Bourtchuladze et al. 1994; Silva et al. 1998; Tronson and Taylor 2007; Lee et al. 2008). The reconsolidation process may serve to strengthen or renew the original fear memory (Nader 2003; Tronson and Taylor 2007). Synaptic plasticity is thought to underlie memory formation. Recent studies have shown that the learning process induces long-term potentiation (LTP) (Rogan et al. 1997; Rioult-Pedotti et al. 2000), a form of synaptic plasticity, and, conversely, that LTP is necessary for memory formation (Rodrigues et al. 2004). Similar to LTM, LTP requires protein and RNA synthesis for its prolonged maintenance, late LTP (L-LTP) (Frey et al. 1988; Abraham et al. 1993; Nguyen et al. 1994; Fukazawa et al. 2003).

In order to understand the molecular mechanism of LTM, we previously isolated a number of neuronal activity-dependent genes, including *activin βA*, *ubiquitin C-terminal hydrolase*, *vesl-1S/homer-1a*, and *SCRAPPER* (Inokuchi et al. 1996; Hegde et al. 1997; Kato et al. 1997; Yao et al. 2007; Okada et al. 2009). *Activin βA*, a member of the TGF-β superfamily (Massague 1998), is one of the genes whose expression is up-regulated following L-LTP induction (Andreasson and Worley 1995; Inokuchi et al. 1996). Activin is a multifunctional ligand that regulates the proliferation and differentiation of numerous cell types (Mather et al. 1997; Ying et al. 1997). Activin binds to the serine/threonine kinase receptor activin type II (ActRII) that is located on the cell membrane (Pangas and Woodruff 2000). Once ligand is bound, the type II receptor recruits and phosphorylates an activin type I receptor (ActRI). Following stimulation by activin, the transcription factors Smad2 and Smad3 are phosphorylated by ActRI

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Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.16659010.

(Pangas and Woodruff 2000). Activin receptor ActRII is highly expressed in the forebrain region (Cameron et al. 1994; Funaba et al. 1997), and its scaffold protein ARIP/S-SCAM is also localized to the synaptic region (Hirao et al. 1998; Shoji et al. 2000).

Recent studies reveal that activin regulates the morphology of dendritic spines (Shoji-Kasai et al. 2007) and neurogenesis (Ageta et al. 2008; Abdipranoto-Cowley et al. 2009; Sekiguchi et al. 2009), has a neuroprotective function (Tretter et al. 2000), and plays a role in anxiety-related behavior (Dow et al. 2005; Ageta et al. 2008; Zheng et al. 2008). We previously found that activin increases the number of synaptic contacts and the length of dendritic spine necks by modulating spinal actin dynamics (Shoji-Kasai et al. 2007). We hypothesized that activin modulates spine actin dynamics and that this in turn affects LTP persistence. Furthermore, activin induces phosphorylation of the *N*-methyl-D-aspartate receptor (NMDAR), a key component in the formation of LTP (Nicoll and Malenka 1999), and increases Ca^{2+} influx through NMDAR (Kurisaki et al. 2008). This activin-induced NMDAR activation persists for >24 h. These results suggest that activin has an important role in the formation of L-LTP and LTM.

Results

Activin is indispensable for in vivo L-LTP

We examined the hippocampal dentate gyrus LTP of urethane-anesthetized rats. A strong high-frequency stimulation (HFS, five 400-pulse trains at 400 Hz) produced a long-lasting L-LTP in vivo that persisted for 24 h (Fig. 1A). However, when the activin inhibitor follistatin (0.5 μg) (Nakamura et al. 1990; Sugino et al. 1997) or an anti-activin A antibody (0.6 μg) was pre-injected into the lateral ventricle, the strong HFS induced an LTP that

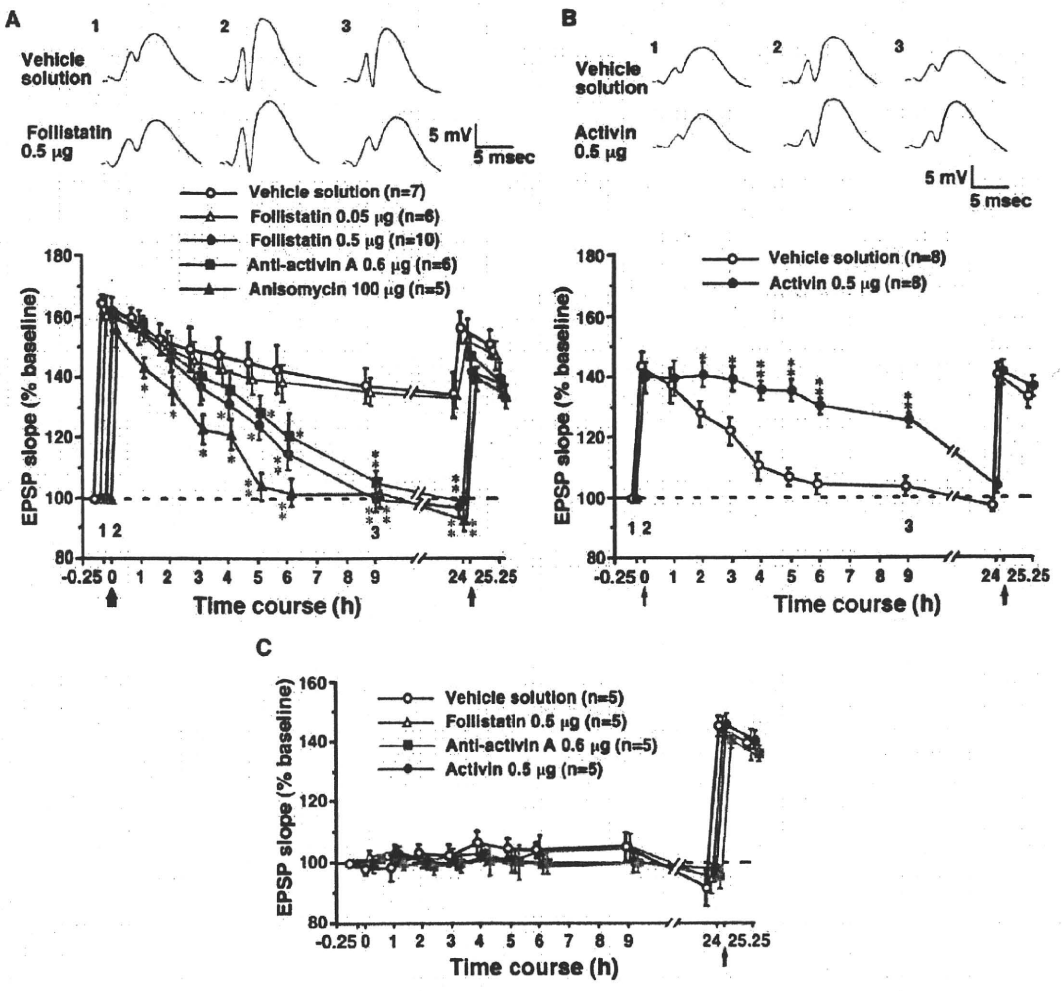

Figure 1. Activin is required for the maintenance of dentate gyrus L-LTP in vivo. (*A*) Effect of follistatin, anti-activin A, and anisomycin on LTP persistence. A strong HFS was delivered at time 0 (thick arrow). (*B*) Effect of activin on LTP persistence. A weak HFS was delivered at time 0 (thin arrow). (*C*) Basal synaptic transmission was not affected by follistatin, anti-activin A, or activin. The average of the fEPSP slope during the 15 min prior to time 0 served as the baseline (100%) for all trials. (*) $P < 0.05$, (**) $P < 0.005$ between the vehicle and experimental groups as determined by one-way ANOVA followed by Fisher's LSD test. At the end of each experiment, a second HFS (100 pulses at 100 Hz) was delivered (red arrows at 24 h). (*Top* panels in *A,B*) Typical fEPSP traces evoked at the times (1, 2, or 3) indicated in each graph. Error bars indicate SEM.

decayed rapidly and returned to basal levels by 9 h (Fig. 1A). The decay time course is similar to that of animals injected with the protein synthesis inhibitor anisomycin (Fig. 1A). In contrast, follistatin or anti-activin A had no significant effect on the initial amplitude and early maintenance of LTP (E-LTP). The effect of follistatin was dose-dependent because the injection of a lower dose (0.05 μg) did not alter the maintenance of L-LTP (Fig. 1A). To exclude the possibility that the LTP decay caused by follistatin or the anti-activin A antibody was due to irreversible damage to hippocampal neurons, a second HFS (100 pulses at 100 Hz) was applied to the same pathway at the end of each experiment (24 h). We observed an enhancement of the field excitatory postsynaptic potential (fEPSP) slope in all animals (Fig. 1A), which eliminates the possibility that neuronal damage occurred.

In a complementary experiment where activin was preinjected into the lateral ventricle, we used a weak HFS (a 50-pulse train at 100 Hz). The weak HFS alone elicited E-LTP that returned to the basal level by 6 h (Fig. 1B). However, pre-administration of activin facilitated the maintenance of LTP, since the weak HFS under these conditions produced an LTP that lasted >9 h. Injection of follistatin, anti-activin A, or activin into the lateral ventricle had no significant effect on basal synaptic transmission (Fig. 1C).

When injected 1 h after the delivery of the weak HFS, activin still enhanced E-LTP (Supplemental Fig. S1A). However, activin failed to facilitate LTP maintenance when administered 3 h after the weak HFS. Consistent with this result is the observation that the inhibitory effect of follistatin on L-LTP establishment was observed when it was injected 1 h, but not 3 h, after the delivery of the strong HFS (Supplemental Fig. S1B).

Generation of activin and follistatin transgenic mice using a forebrain-specific Tet-OFF system

To examine the role activin plays in fear memory formation, activin activity was genetically suppressed or increased in the forebrain of transgenic mice carrying a Tet-OFF system. In this system, the tetracycline-controlled transactivator (tTA) is under the control of the CaMKIIα promoter to achieve forebrain-specific expression (Fig. 2A; Mayford et al. 1996). We generated two distinct lines of responder mice, ABI and FBI, in which activin and follistatin, respectively, were controlled by the tetracycline response element (TRE) promoter. Double (FBItTA and ABItTA)-transgenic mice were obtained by heterozygous crossings. A diet containing DOX (6 mg/g food) was fed to pregnant mice for 7 consecutive days immediately prior to the expected date of confinement to reduce leak expression of follistatin or activin during the late embryonic stage. Double-transgenic ABItTA and FBItTA mice were fertile, bred normally, and maintained a normal body weight under the no-DOX treatment.

The tTA binds specifically to the TRE promoter and activates transcription in the absence of DOX (Mayford et al. 1996). In these mice, TRE bidirectionally regulated LacZ (Fig. 2A). Thus, LacZ activity was detected in some brain regions including the hippocampus, striatum, and amygdala in ABItTA, but not ABI, mice in the absence of DOX treatment. LacZ activity was also detected in some brain regions including the hippocampus and cortex in FBItTA, but not FBI, mice in the absence of DOX treatment (Fig. 2B; Supplemental Fig. S2). An ELISA analysis revealed that the activin level in the hippocampus of ABI mice (0.06 ng/mg protein, Fig. 2C) was equivalent to that of wild-type mice (Ageta et al. 2008), showing no leaky transgene expression in ABI mice. Moreover, the follistatin level in the hippocampus of FBItTA mice (0.25–0.5 ng/mg protein) was sufficient to antagonize the endogenous hippocampal activin (0.06 ng/mg protein) in the absence of DOX treatment (Sugino et al. 1997). DOX administration decreased the ectopic activin and follistatin to basal levels within 3 d in the hippocampus of ABItTA and FBItTA mice (Fig. 2C). Suppression by DOX was reversible and almost completely recovered within 3 d for FBItTA and 14 d for ABItTA (Fig. 2C). There was no significant difference in the activin level in the hippocampus of ABI and ABItTA mice on days 1 and 7 (Fig. 2C). No transgene expression was observed in the cerebellum and medulla of ABItTA and FBItTA mice in the absence of DOX treatment.

The maintenance of L-LTP is reduced in FBItTA mice

Hippocampal slices were prepared from 6- to 10-wk-old FBI and FBItTA mice, and fEPSPs were recorded in the hippocampal CA1 region after the application of single test stimulus every 20 sec (0.05 Hz) to the Schaffer collaterals. We first examined whether the basic property of excitatory synaptic transmission was normal in FBItTA mice. We measured the initial slope of fEPSP in order to quantify the strength of the synaptic response. The input–output relationship in FBItTA mice was similar to that of FBI mice (Fig. 3A). In the CA1 region, paired stimulation with short intervals usually causes paired-pulse facilitation (PPF). The extent of PPF with interpulse intervals of 25–500 msec of FBItTA mice was identical to that of FBI mice (Fig. 3B). These results suggest that there are no physiological differences in the basic properties of excitatory synaptic transmission between FBItTA and FBI mice.

We then examined whether E-LTP in the CA1 region was altered in FBItTA mice. After a baseline recording of at least 15 min, LTP was induced by tetanic stimulation. A single train of tetanic stimulation, 100 Hz for 1 sec, elicited identical E-LTP in both strains of mice (136.2 ± 14.0% and 144.8 ± 16.0%, 90–120 min after tetanic stimulation in FBItTA and FBI mice, respectively). Three trains of tetanic stimulation at 20-sec intervals caused much larger E-LTP than that after a single train of tetanus, but E-LTP in FBItTA mice was similar to that in FBI mice (161.8 ± 9.3% and 175.8 ± 8.0%, 90–120 min after tetanic stimulation in FBItTA and FBI mice, respectively; Fig. 3C). Thus, E-LTP was unaffected in FBItTA mice.

In contrast, L-LTP in FBItTA mice was markedly reduced as compared with the robust L-LTP observed in FBI mice (Fig. 3C). The slope of fEPSP 3–3.5 h and 5–5.5 h after tetanic stimulation in FBItTA mice was 148.6 ± 11.0% and 139.0 ± 10.2%, whereas that in FBI mice was 174.6 ± 10.2% and 167.6 ± 13.2%, respectively ($P < 0.05$). The fEPSPs elicited by stimulation of the second pathway to activate an independent set of Schaffer collaterals were unaltered throughout the experiment in both strains of mice. To clarify the biochemical effect of follistatin transgene expression, we analyzed the phosphorylation level of Smad 2/3 in the hippocampus of FBI and FBItTA. Phosphorylation of Smad 2/3 was significantly decreased in the hippocampus of FBItTA but not FBI (Supplemental Fig. S3). These results demonstrate that L-LTP was reduced in FBItTA mice, whose follistatin levels had an ability to suppress endogenous activin activity in the hippocampus.

Anxiety levels of ABItTA and FBItTA

Activin has multiple roles in the brain; for example, it influences anxiety-related behavior (Dow et al. 2005; Ageta et al. 2008; Zheng et al. 2008), modulates postnatal neurogenesis (Ageta et al. 2008), and protects neurons from ischemic damage (Tretter et al. 2000). In our previous study, we generated ACM4 and FSM transgenic mice in which activin and follistatin, respectively, were overexpressed in a forebrain-specific manner under the control of the αCaMKII promoter. FSM mice exhibited enhanced anxiety compared with wild-type littermates, while ACM4 mice showed reduced anxiety (Ageta et al. 2008). Therefore, we performed two behavioral analyses, such as a light and dark test and

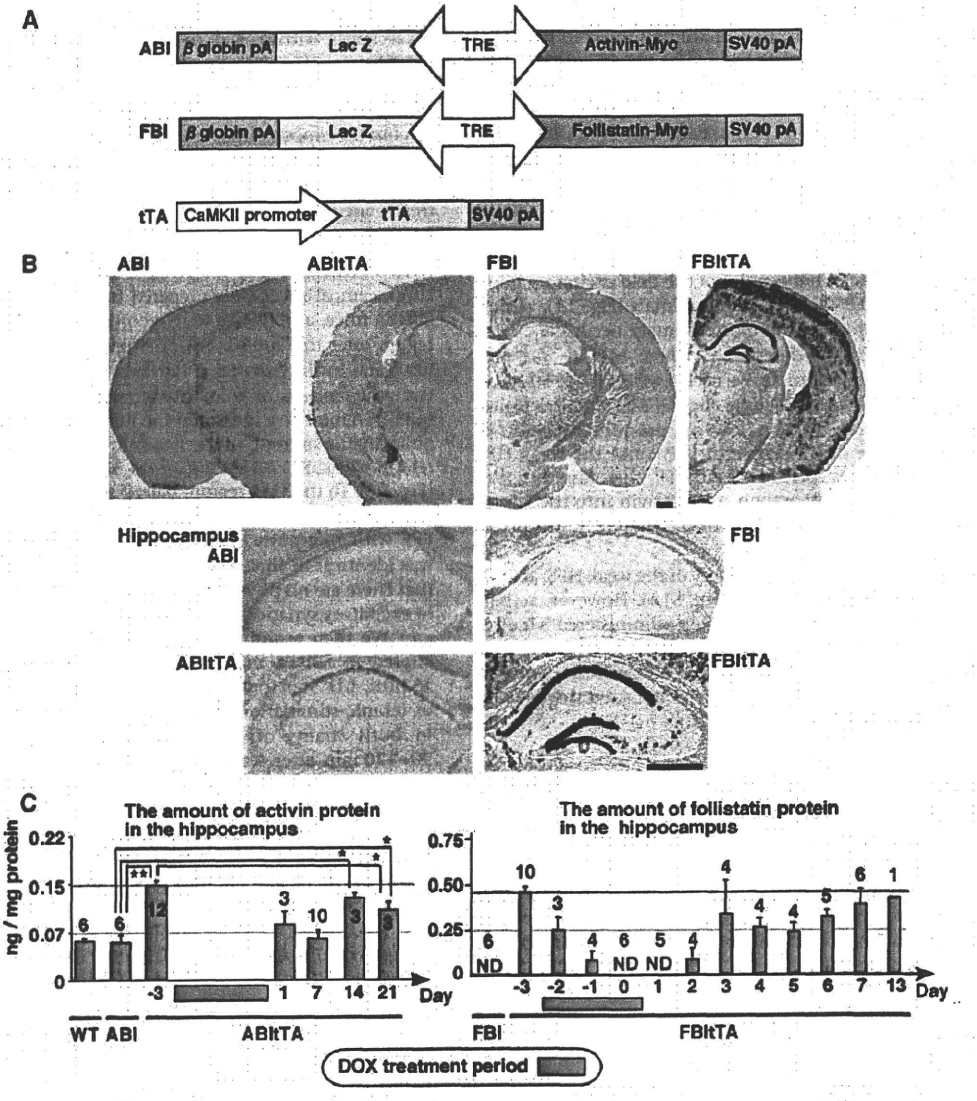

Figure 2. Generation of activin and follistatin transgenic mice using a forebrain-specific Tet-OFF system. (*A*) Schematic transgene representations of ABI (*upper*), FBI (*middle*), and tTA (*lower*) constructs. (TRE) Tetracycline response element, (tTA) tetracycline-controlled transactivtor, (pA) polyadenylation signal. (*B*) X-gal staining. (*Upper* row) Coronal brain sections from ABI, ABItTA, FBI, and FBItTA mice (10–15 wk old). (*Lower* row) Enlarged images of the hippocampus. Scale bars, 500 μm. (*C*) Measurement of activin and follistatin level using an ELISA assay with anti-activin and anti-follistatin antibodies. (Red line) Maximal levels of activin (*left* panel) or follistatin (*right* panel) in the hippocampus in the absence of DOX (10–15 wk old). Mice were fed DOX for 3 consecutive days (from noon of day −3 to noon of day 0, orange bars). The animals were then sacrificed and the hippocampus was dissected out in the afternoon of the day indicated and used for the ELISA. (ND) Not detected. The number *above* each bar indicates the number of animals used. Error bars indicate the SEM. (*) $P < 0.05$, (**) $P < 0.001$ compared with activin in the hippocampus of ABI mice in the absence of DOX, as determined by one-way ANOVA followed by Fisher's LSD test.

risk-taking behavior test, to examine the anxiety levels of FBItTA and ABItTA mice. In a risk-taking behavior test the amount of time spent in the center of an open field strongly correlates with an animal's level of anxiety (Ageta et al. 2008). The double-transgenic FBItTA and ABItTA mice showed normal anxiety-like behavior in the light–dark and risk-taking behavior tests (Supplemental Fig. S4A,B), suggesting that these mice show normal responses compared with the mutant mice used in previous work (ACM and FSM, respectively; Ageta et al. 2008). Furthermore, the performance of the double-transgenic mice (ABItTA and FBItTA) was comparable to single-transgenic mice (ABI and FBI) in sensitivity to electric footshock (Supplemental Fig. S4C). Neurogenesis in the adult hippocampus was reduced in FBItTA mice, but the reduction was less severe than in FSM mice (Supplemental Fig. S5). Therefore, the observed reduction in neurogenesis in FBItTA mice has no influence on the anxiety level. The differences in anxiety phenotype may be, perhaps, due to different follistatin levels in the brain. The FSM mice exhibited a high level of follistatin expression compared with FBItTA mice (Ageta et al. 2008).

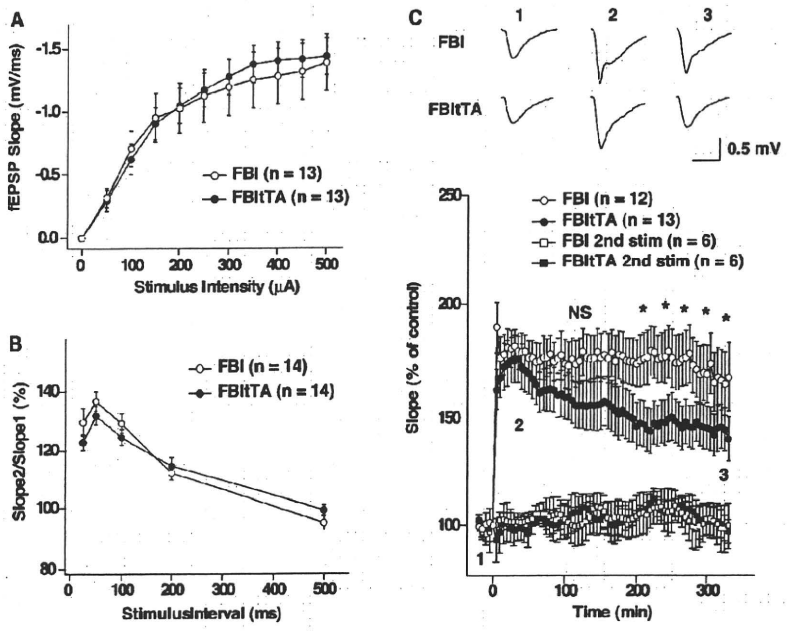

Figure 3. The maintenance of CA1 L-LTP in hippocampal slices from FBItTA mice is reduced as compared with FBI mice. (*A*) Input–output curve of fEPSP slope (millivolt [mV]/millisecond [msec]) versus stimulus (microampere [μA]) at the Schaffer collateral–CA1 pyramidal cell synapse in FBItTA and FBI mice. Data are presented as means ± SEM. (*B*) Paired-pulse ratio of fEPSPs in FBItTA and FBI mice. The initial slope of the fEPSP was measured in order to quantify the strength of the synaptic response. The ratio of the second to first response is plotted against the interstimulus interval. Data are presented as means ± SEM. (*C*) E-LTP and L-LTP elicited by three 100-Hz trains spaced at 20-msec intervals in FBItTA and FBI mice. The fEPSP slopes elicited by stimulation of the second independent pathway at 0.017 Hz were stable throughout the experiments. (*Insets*) Representative fEPSP traces taken at the times indicated on the graphs in C. (*) $P = 0.05$, (NS) not significant.

Once-consolidated fear memory is weakened by follistatin overexpression during the retrieval phase

We performed contextual fear-conditioning tests on FBItTA and FBI mice in the absence of DOX (Fig. 4, Experiment A). A 1-d retention test showed a significant reduction in the freezing response in FBItTA mice in Test-1 (1 d after conditioning) and Test-2 (2 d after conditioning) compared with FBI mice. There was no significant genotype effect on STM formation (Fig. 4, Experiment B). These results are consistent with the requirement for activin in L-LTP (Figs. 1A, 3C). When transgene expression was turned off by administration of DOX (orange bars, Fig. 4) for 3 consecutive days prior to conditioning, the 1-d memory of FBItTA mice was normal (Fig. 4, Experiment C, Test-1). This result indicates that follistatin expression during development of FBItTA mice did not affect the formation of neuronal circuits that are involved in contextual fear conditioning. Thus, LTM consolidation, but not short-term memory, requires activin activity in the forebrain.

One-week memory tests measured a comparable freezing behavior in FBItTA and FBI mice when DOX was administered for 3 consecutive days before conditioning (Fig. 4, Experiment D, Test-1 [indicated as T1]; Test-1 was performed 7 d after conditioning). Thus, the 1-wk memory was normal despite the inhibition of activin signaling during maintenance and retrieval, from day 3 to day 7, when fear conditioning was carried out in the absence of transgene expression. Importantly, when the same animals were retested for freezing behavior 24 h later, the FBItTA mice showed significantly less freezing response compared with the FBI mice (Fig. 4, Experiment D, Test-2 [T2]; Test-2 was performed 8 d after conditioning). In contrast, there was no difference in freezing between FBItTA and FBI mice in Test-2 of Experiment C. In these two paradigms (Experiments C and D), the interval between conditioning and Test-2 was the same (8 d). One of the major differences in the experimental paradigm was that in Experiment D the forebrain activin signal was inhibited during the retrieval period (Test-1), while in Experiment C it was not. We performed an additional experiment that is based on Experiment D. In this paradigm, mice were continuously fed on DOX to suppress the follistatin expression during the maintenance and retrieval phase (Experiment F). We did not observe a significant genotype effect in either Test-1 or -2 of Experiment F. Thus, inhibition of the activin signal during retrieval may result in significant suppression of the subsequent freezing response.

To examine this hypothesis, conditioned animals were reexposed to the conditioning chamber for 1 min at 4 d after the conditioning, a time at which forebrain activin was blocked (Fig. 4, Experiment E). In this experiment, we used short time reactivation because long time reactivations induce extinction (Suzuki et al. 2004). The other procedures were essentially the same as in Experiment D. We observed that injection of the protein synthesis inhibitor anisomycin 30 min after the reactivation into wild-type mice, which had been subjected to the same experimental paradigm as in Experiment E, resulted in a reduction in the freezing response in Test 24 h later (Supplemental Fig. S6). In experiment E, we observed a significant reduction in freezing level in Test-1 (7 d after conditioning) of FBItTA mice compared with FBI mice, strongly suggesting that the reactivation of fear memory in the absence of forebrain activin activity caused the decreased freezing response.

Fear memory is influenced by activin overexpression during the retrieval phase

A complementary experiment with ABItTA and ABI mice strengthens the idea that forebrain activin is important for proper processing of fear memory following memory reactivation (retrieval) (Fig. 5, Experiment G). In this experiment, we used a relatively weak conditioning protocol to avoid saturation of freezing response. A 3-wk memory test showed a normal freezing response in ABItTA mice compared with ABI mice (Test-1 in Experiment G, 21 d after conditioning), when the activin level was reduced to the basal level at conditioning by DOX administration. However, when the freezing response of the animals was tested 24 h later, ABItTA mice showed significantly more freezing than ABI mice (Test-2 in Experiment G, 22 d after conditioning). Furthermore, we observed a significant increase in freezing level in Test (21 d after conditioning) of ABItTA compared with their

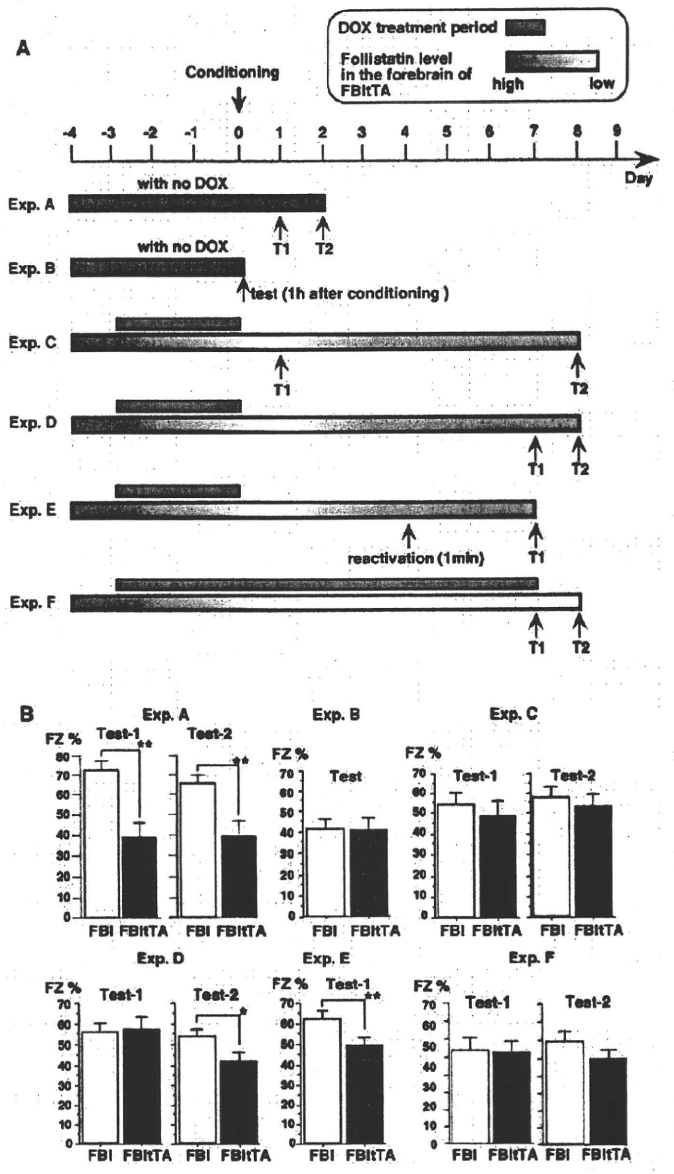

Figure 4. Once-consolidated fear memory is weakened by follistatin overexpression during the retrieval phase. (*A*) Experimental schedule. The horizontal axis indicates the time line. Mice were fed DOX for 3 consecutive days before conditioning (from noon of day −3 to noon of day 0), as indicated (orange bars). The density of blue color indicates the predicted level of follistatin in the forebrain. The numbers of mice used were: Experiment A (FBItTA, $n = 8$; FBI, $n = 9$); Experiment B (FBItTA, $n = 10$; FBI, $n = 10$); Experiment C (FBItTA, $n = 8$; FBI, $n = 11$); Experiment D (FBItTA, $n = 10$; FBI, $n = 10$); Experiment E (FBItTA, $n = 17$; FBI, $n = 13$); Experiment F (FBItTA, $n = 11$; FBI, $n = 11$). In all the experimental paradigms, conditioning was performed in the afternoon of day 0 with the same footshock protocol. (T1) Test-1, (T2) Test-2. (*B*) Freezing response during the test period. (FZ%) Average freezing percentage during the 6-min test period. (*) $P < 0.05$, (**) $P < 0.001$, statistically significant differences between FBItTA and FBI mice, as determined by one-way ANOVA followed by Fisher's LSD test. Error bars indicate SEM. Experiment C: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,17)} = 0.39$, $P = 0.539$; Test effect, $F_{(1,17)} = 2.32$, $P = 0.146$; genotype × Test, $F_{(1,17)} = 0.03$, $P = 0.8629$. Experiment D: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,17)} = 0.71$, $P = 0.4$; Test effect, $F_{(1,17)} = 10.16$, $P = 0.0054$; genotype × Test, $F_{(1,17)} = 6.58$, $P = 0.02$. Experiment F: Two-way repeated-measures ANOVA, genotype effect, $F_{(1,20)} = 0.57$, $P = 0.46$; Test effect, $F_{(1,20)} = 0.10$, $P = 0.75$; genotype × Test, $F_{(1,20)} = 2.92$, $P = 0.103$.

littermates (ABI and wild-type mice) when conditioned animals were reexposed to the conditioning chamber for 1 min at 17 d after conditioning, a time at which forebrain activin was increased in ABItTA (Fig. 5, Experiment H). Taken together, these results indicate that the functional activin level in the forebrain during fear memory retrieval (in this case, Test-1 in Experiment G and reactivation in Experiment H) determines the later freezing response (Test-2 in Experiment G and Test in Experiment H).

Discussion

In this study we showed that activin is indispensable for the late maintenance of hippocampal dentate gyrus L-LTP in vivo and CA1 L-LTP in slice preparation. In the marine snail *Aplysia*, TGF-β induces long-term, but not short-term facilitation at the synapses between the sensory and motor neurons (Zhang et al. 1997). Furthermore, in rat cultured hippocampal neurons, treatment with TGF-β2, another isoform of TGF-β, affected synaptic strength and induced phosphorylation of CREB (Fukushima et al. 2007). Thus, the TGF-β family of proteins, namely, activin and TGF-β 1/2, participate not only in development but also in the neuronal plasticity of the mature CNS. In addition, we revealed the existence of prolonged E-LTP in the dentate gyrus, which on the one hand differs from E-LTP in its longer persistence and activin dependency; and on the other differs from L-LTP in its shorter persistence and lack of requirement for protein synthesis. This prolonged E-LTP has been previously described as an intermediate phase LTP (I-LTP) to occur in area CA1 of the hippocampus (Winder et al. 1998). It was found to differ from E-LTP and L-LTP in its molecular mechanisms since it is dependent on protein kinase A, does not require protein synthesis, and is suppressed by calcineurin overexpression. Although it is not clear whether dentate gyrus prolonged E-LTP and CA1 I-LTP share the same molecular mechanisms, it appears that temporally distinct tri-phase LTP is a common characteristic of hippocampal LTPs.

We demonstrated that activin in the brain is required for formation of L-LTP and consolidation of LTM (Figs. 1, 3, and 4). Follistatin failed to suppress L-LTP maintenance when it was administered 3 h after a strong HFS (Supplemental Fig. S1), and this result is consistent with the behavioral analysis of FBItTA mice. After the acquisition phase, the ectopic expression of follistatin in the maintenance phase did not affect LTM formation (Fig. 4, Experiment D, Test-1), indicating that the presence of follistatin in the maintenance phase has no effect on either L-LTP or LTM. Thus, our results strengthen the correlation between L-LTP and LTM.

There are several mechanisms by which activin may participate in L-LTP and LTM. Activin modulates dendritic spine morphology and increases the number of synaptic contacts (Shoji-Kasai et al. 2007). Activin potentiates NMDA receptor-mediated signaling cascades for long periods of time (Muller et al. 2006; Kurisaki et al. 2008). In